Susceptibility of the Imported Cabbageworm to Bacillus thuringiensis Berliner[1, 2]

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The infectivity of *Bacillus thuringiensis* Berliner for *Pieris rapae* (L.) was tested at the Laboratory of Insect Pathology, Department of Biological Control, University of California in Berkeley, California. The strain of *B. thuringiensis* used in the present study was furnished by Dr. E. A. Steinhaus. It is believed to be the same strain that Mattes (1927) had isolated and described from the diseased larvae of the Mediterranean flour moth.

Bacillus thuringiensis was first isolated from the larvae of the Mediterranean flour moth, *Ephestia kühniella* Zeller, by Berliner (1911). In 1915, Berliner described the species under this name, and called the bacterial disease "flaccidity." A thorough taxonomic discussion of this species was presented by Steinhaus (1951). Although *B. thuringiensis* Berliner might be considered as a strain of *Bacillus cereus* Frankland and Frankland, a common soil bacterium, Steinhaus (1951) suggested that because of its characteristic and persistent pathogenicity for insects, the name should be retained and, at least for the time being, considered as distinct from *B. cereus*.

Several insect species are susceptible to *B. thuringiensis*. Both Berliner (1915) and Mattes (1927) observed that the larvae of Mediterranean flour moth were highly susceptible to the bacillus. However, larvae of other Lepidoptera, such as certain species of *Nygmia, Lymantria,* and *Thaumetopoea*, and both larvae and adults of certain Coleoptera, such as *Calandra, Tribolium, Dermestes, Tenebrio,* and *Gnathocerus,* were not susceptible according to Berliner (1915). In 1924, Shepherd mentioned that the bacillus was used in Germany to control the cosmopolitan bran beetle *Echocerus cornutus* (Fabr.). Many workers had shown that *B. thuringiensis* (= *B. ephestiae*) was highly pathogenic to corn borer larvae (Metalnikov and Chorine 1929, Metalnikov, Hergula, and Strail 1930, 1931, Chorine 1931, Husz 1931).

Steinhaus (1951) stated that Metalnikov and Chorine in 1929 found the bacillus to be highly virulent for larvae of *Porthetria dispar* (L.), *Aporia crataegi* L., and *Vanessa urticae* (L.), but not for certain grasshoppers, mosquitoes, and beetles. In an extensive study with spore-forming bacteria in 1951, Steinhaus reported that none of the nine strains of

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Bacillus cereus which were fed to five species of test insects showed any consistent degree of pathogenicity. However, *B. thuringiensis* was highly virulent to *Colias philodice eurytheme* Boisd., moderately virulent to *Junonia coenia* Hübner, *Phryganidia californica* Pack., and *Prodenia praefica* Grote, and least virulent to *Peridroma margaritosa* (Haworth). He also found that a bacillus isolated from a diseased larva of *Aphomia gularis* (Zeller) was highly virulent to *J. coenia, P. praefica,* and *C. philodice eurytheme,* but less so to *P. californica,* and only slightly if at all to *P. margaritosa.* This bacillus was identified by N. R. Smith as *Bacillus thuringiensis.* The sod webworm, *Crambus bonifatellus* (Hulst), was found to be susceptible to *B. thuringiensis* by Hall (unpubl.).

Methods of Study.

The cabbageworms were reared during the summer from eggs laid by field collected adults, and during the winter from eggs laid by laboratory reared adults. The eggs were disinfected by immersing for 90 minutes in a solution of 10 per cent formaldehyde, by weight, according to the method described by Thompson and Steinhaus (1950). After sterilization, the eggs were washed in running tapwater for about 15 minutes to remove the formaldehyde. Then they were placed in sterile half-pint cardboard containers with a vial containing a leaf of either mustard or kale. After the larvae had reached the second instar, they were placed by pairs in sterile containers until they were ready to be used.

Depending on the availability of the cruciferous plants, the cabbageworms were fed either the chicken kale, *Brassica oleraceae* var. *acephala* DC., the smooth leaved mustard, *B. campestris* L., or the rugose leaved mustard, *B. Kaber* (DC.) Wheeler (= *B. arvensis* sensu Rabenh.). In both the treated and control groups, the larvae were reared individually in sterile containers.

Symptoms and Pathology.

In an initial pathogenicity test, 11 fourth instar imported cabbageworms were fed mustard leaves which had been dipped in a suspension of spores of *B. thuringiensis.* Ten fourth instar larvae served as controls. Nine of the treated larvae died during the first 2 days, one on the fourth, and one on the sixth day after infection. All the dead larvae contained large motile rods. In the control group, bacteria other than *B. thuringiensis* apparently caused the death of two larvae.

Two additional tests were conducted with larvae in the early and late fifth instars. In the first trial, 30 early fifth instar larvae were fed leaves that had been dipped in a suspension of spores in blood albumin solution. Thirty untreated larvae of the same age served as controls. During the first 2 days, 23 of the larvae which had been fed treated leaves died, and the remainder died by the fifth day. None of the control larvae died.

The second test was conducted with 20 late fifth instar larvae, which were fed the bacterial spores, and 20 larvae of the same age for the controls. Ten of the 20 control larvae were fed the blood albumin solution and 10 left untreated. None of the control larvae died. All the treated larvae died except for one larva which pupated without feeding on the spores. Sixteen of the treated larvae died within 2 days, and the remainder died by the fifth day.

The results indicate that *B. thuringiensis* is highly virulent to the imported cabbageworm. Even larvae which feed on the bacterial spores only a day or two before pupation may die from infection. Berliner (1915), however, reported that in *E. kühniella,* if the larvae pupated within 2 days from the time of infection, they were not affected by the disease. The period of infection[3] of the bacterial disease in *P. rapae* is very short, only 1 to 5 days.

According to Steinhaus (1951), the susceptible larvae of most lepidopterous species show similar symptoms following infection by *B. thuringiensis.* The first signs of infection in the imported cabbageworm were a loss of appetite and a decreased response to tactile stimulation. Some larvae showed signs of diarrhea as indicated by the dirty anal segments and by the tendency to stick to the sides of the rearing container by their terminal segments. Others vomited some fluid. Occasionally a dark area or spot appeared on the back or sides of the larva. The larva usually died in an extended flaccid position, but some showed signs of contraction and a tendency to curve backwards. Shortly after death, the body darkened from green to dark brown or black. This discoloration is attributed to secondary organisms (Berliner 1915, Steinhaus 1951). The body contents were soft and somewhat fluid, and the integument was generally firm.

The pathogenesis or course of infection as it takes place within the imported cabbageworm was studied at three different stages. Longitudinal sections were cut from five larvae in the early stage of bacterial infection, from two larvae in the middle to late stages, and from three larvae soon after their death.

Shortly after the spores of *B. thuringiensis* were ingested by the larva, the portion of the mid-gut epithelium that lies posterior to the peritrophic membrane became disorganized (Fig. 1A). Some of the epithelial cells became detached from the wall of the mid-gut. Hemorrhage occurred at the sites where the epithelium was lost, and the blood cells accumulated in the lumen near the exposed intestinal muscles (Fig. 1B). This damage to the mid-gut probably caused the loss of the larva's appetite. At this stage, few vegetative cells of the bacteria were visible in the lumen of the gut. In the larva of the Mediterranean flour moth, Berliner (1915) observed similar destruction of the mid-gut epithelium before the bacteria had increased in numbers, but Mattes (1927) claimed that the bacteria multiplied rapidly within the lumen before the epithelium was damaged.

The bacteria penetrated into the body cavity apparently at the sites where the mid-gut epithelium was destroyed. Berliner (1915) found the same type of penetration in *Ephestia,* but Mattes (1927) believed that this was true only in a very few instances. In *Ephestia,* the bacteria started to enter the hemocoele through the anterior portion of the mid-gut according to Mattes (1927), but in the imported cabbageworm, the bacteria appeared to enter the hemocoele near the posterior region of the

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[3] The period of infection is defined as the interval from the time the host feeds on the pathogen until the death of the host from a frank infection.

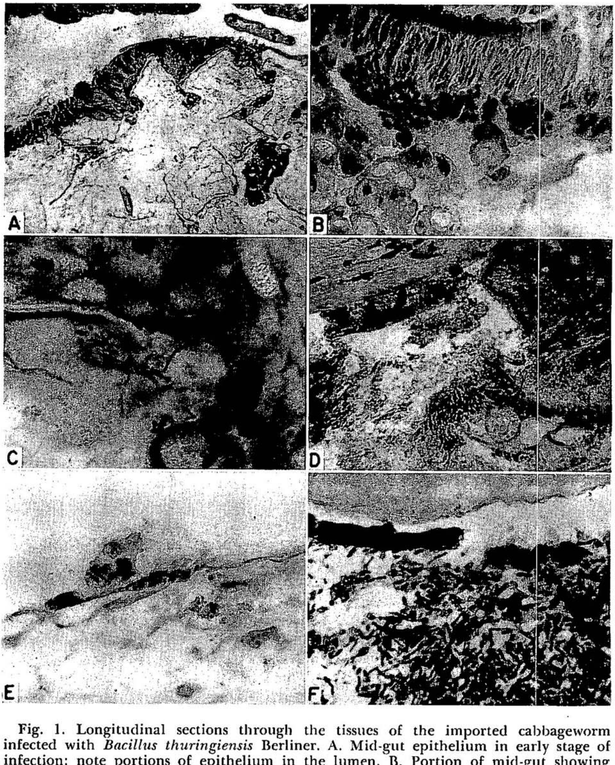

Fig. 1. Longitudinal sections through the tissues of the imported cabbageworm infected with *Bacillus thuringiensis* Berliner. A. Mid-gut epithelium in early stage of infection; note portions of epithelium in the lumen. B. Portion of mid-gut showing cluster of blood cells outside of circular muscle layer where the epithelial cells had fallen off; observe in the lower left corner, the loose cells in the lumen. C. Group of bacilli entering the hemocoele in a region where the mid-gut epithelium had been destroyed; lower left corner is the lumen of the gut. D. Bacilli entering the hemocoele after the mid-gut epithelium has been destroyed. E. Mass of bacteria embedded in the thin remnant of mid-gut wall. F. Intense septicemia; note the hypodermis beginning to break down. A, B, C, D, E, stained with Mayer's acid hemalum and eosin; F, with iron hematoxylin and eosin. A, taken under low power; B, D, under high power; C, E, F, under oil immersion.

mid-gut (Fig. 1C, D). No bacteria were observed entering the body cavity through the gaps between the epithelial cells as reported by Mattes (1927) for *Ephestia*.

As the infection advanced, the destruction of the mid-gut wall progressed anteriorly until most of the epithelium was destroyed. In place of the epithelium, the damaged portions of the mid-gut were lined with a thin necrotic tissue apparently derived from the muscle and neighboring cells (Fig. 1E). The walls of the fore- and hind-guts remained intact until a late stage of infection.

The bacteria multiplied rapidly near the damaged mid-gut and apparently penetrated between the muscle cells into the body cavity. As in the case of *Ephestia* (Mattes 1927) the penetration of the bacteria into the epithelial cells themselves was not observed.

Once the bacillus was in the hemocoele, it increased rapidly in the blood and caused an intense septicemia. Even at this stage, the number of bacteria in the lumen of the mid-gut was not great. When the bacteria entered the hemocoele, the fat tissue was among the first tissues to disintegrate. Mattes (1927) reported that in *Ephestia* the visible damage of the organs did not become apparent until the blood was heavily infected with bacteria, at which time the external symptom of "flaccidity" appeared.

At a very late stage of infection or soon after death, only remnants of the mid-gut epithelium were left. There was an abundance of bacteria in the hemocoele (Fig. 1F) and the lumen of the gut. Besides the fat, other tissues and organs, such as the muscle, hypodermis, trachea, salivary gland, and Malpighian tubules, showed distinct signs of pathology. In general, the cytoplasm of the cells was stained faintly but the nucleus which was usually small, was stained intensely, especially around the margins. The peritrophic membrane became thickened with an occasional mass of bacteria embedded in it.

In *P. rapae,* the breakdown of the tissues apparently took place from the surface that was in contact with the blood. There appeared to be a progressive liquefaction of the surface of the cells and an alteration of the cell membranes. The bacteria might penetrate into the cells before the cells completely broke down.

Among the last tissues to disintegrate were the muscles and nerves. The muscle fiber first lost its typical striated pattern, then became short, broad and irregular, and finally disintegrated. Masses of bacteria penetrated the muscles shortly thereafter. The nervous tissues, especially the ganglia, were also penetrated by bacteria at a late stage. After death, only a few remnants of internal tissues remained among the immense number of bacteria in the hemocoele.

Median Lethal Dose of Bacterial Spores.

Since *B. thuringiensis* was so highly pathogenic to the imported cabbageworm, a study was undertaken to determine the dosage of bacterial spores required to kill 50 per cent of the fifth instar larvae. The bacterial spores were obtained from a plain nutrient agar slant that had been streaked 3 days earlier with a pure strain of *B. thuringiensis.* A portion of the colony, which had sporulated, was suspended in sterile distilled water by agitating it in a sterile Waring blender. The number of spores in the suspension was determined with a hemocytometer before and after applying the treatments.

A 0.25 ml syringe, manipulated by a micrometer, was used to deliver a measured volume of spore suspension onto discs of mustard leaves measuring one quarter inch in diameter. The drop of spore suspension added to each disc was allowed to dry before it was fed to the larva. The larvae were starved about 5 hours so that they would devour the entire disc before it dried out. They usually ate the entire disc within an hour.

There were three tests. In the first test, five fifth instar larvae were used for each treatment. The treatments consisted of a control and three treated groups of 1, 2, and 3 cubic millimeters of spore suspension. The suspension contained about 8,200 spores per cubic millimeter. Two of the larvae fed the 3 cubic millimeter dosage died from infection with *B. thuringiensis* in 2 days, and one pupa died from a non-spore forming bacterium. In the group treated with 2 cubic millimeters of spore suspension, one larva died in the pupal stage from the bacillus and another died apparently from a non-spore forming bacterium. No larva died in the control or the group treated with 1 cubic millimeter of spore suspension.

The inoculum in the second test contained about 5,000 spores per cubic millimeter. In addition to the control, there were four treatments of 1, 2, 3, and 5 cubic millimeters of spore suspension. Ten fifth instar larvae were used in each treatment. In the treatment with 5 cubic millimeters of spore suspension, two of the 10 larvae died from the bacillus and three from other causes. In the group treated with 2 cubic millimeters of spore suspension, one larva died from the bacillus and one from an unknown cause. The group fed 1 cubic millimeter of spore suspension had one larval death from an unknown cause. None of the larvae died in the control group or in the group treated with 3 cubic millimeters of spore suspension.

The results of the above two trials indicated that the highest dosage of spores used was near the threshold required to kill the larvae. Apparently a concentration of 10,000 to 25,000 spores per larva was needed to cause a lethal infection.

The third test was conducted as in the first two tests, but there were five treatments of 1, 2, 3, 4, and 5 cubic millimeters of spore suspension. Approximately 15,000 spores were present in each cubic millimeter of solution. Each of the five treated groups and the control group contained 10 fifth instar larvae. The larvae were weighed individually on an analytical balance to the nearest milligram. Many of the larvae, after eating only a portion of the disc, lost their appetites and refused to eat for a day or two. Some of these died from bacterial infection but a few developed into adults. The larvae, which died from infection with *B. thuringiensis,* were killed mostly on the third and fourth day, while none died from the bacillus after the fifth day. The results obtained in the third test are presented in Table 1.

At least 50 per cent of the larvae died from the bacillus when they were fed with 2 cubic millimeters and larger quantities of spore suspension. The dosage of spores needed to kill 50 per cent of the fifth instar larvae was about 30,000 spores per larva or approximately 450 spores per milligram of body weight. As mentioned previously, one of the reasons that a 100 per cent mortality was not obtained in the third test with the higher quantities of spores might have been due to the incomplete consumption of the treated discs by the larvae.

Table 1. The number of spores of *Bacillus thuringiensis* Berliner required to kill fifth instar larvae of *Pieris rapae* (L.). Each treatment had 10 larvae.

Volume spore suspension (mm3)	Total number spores	Dosage	Number dead larvae		Total adults
		Av. no. spores mgm body weight	*B. thuringiensis*	Others	
Control	0	0	0	0	10
1	15,000	340	1	1	8
2	30,000	450	5	0	5
3	45,000	660	6	0	4
4	60,000	760	6	0	4
5	75,000	1,340	7	0	3

Summary

The imported cabbageworm, *Pieris rapae* (L.), is highly susceptible to *Bacillus thuringiensis* Berliner. After ingestion of a lethal dose of bacterial spores, the larvae died in 1 to 5 days. The infected larvae exhibited symptoms similar to those described for other lepidopterous larvae infected with *B. thuringiensis.*

The pathogenesis or course of infection of the disease resembled more closely that described by Berliner than that described by Mattes.

Approximately 30,000 bacterial spores per larva, or about 450 spores per milligram of body weight were needed to kill 50 per cent of the fifth instar larvae.

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